ASSOCIATION BETWEEN FUNGAL DETECTION IN THE AIRWAYS AND MODERATE TO SEVERE ASTHMA IN HORSES: A CLINICAL STUDY

Pauline Barbazanges[1], Anne Couroucé[1], Gabin Le Digarcher[1], Jacqueline Cardwell[2], Eliott Schmitt[3], Marie-Pierre Toquet[3], Louise Lemonnier[1], and Eric Richard[3]

[1]Oniris
[2]The Royal Veterinary College Department of Pathobiology and Population Sciences
[3]Universite de Caen Normandie IUT de Caen

May 06, 2024

Abstract

Background: Fungi are ubiquitous in horses' environment. Their contribution to the pathophysiology of severe asthma (SA) is acknowledged, while controversies remain for mild-moderate asthma (MA). **Objectives:** We hypothesized that fungi were a risk factor for asthma. Our objective was to compare different combinations of analytical methods (cytology, culture) and sampling sites (tracheal wash (TW), bronchoalveolar lavage fluid (BALF)) in relation to clinical status (control, MA, SA). **Study design:** Prospective cross-sectional study. **Methods:** The study population included asymptomatic racing horses in the field and horses referred to the hospital for respiratory investigations. Fungi were detected by cytology and identified by mycology on TW and pooled BALF. Chi-square tests were used for prevalence comparison between groups and association with clinical investigations. **Results:** A total of 155 horses (85 MA, 35 SA and 35 controls) were included in the study. The overall proportions of fungal detection in TW ranged from 45.7% to 89.4% among groups. The prevalence of fungal detection in BALF was significantly lower by cytology for SA (5.7%) than MA horses (23.6%) and significantly higher by culture for MA horses (31.8%) than controls (8.6%). Fungal detection by culture in BALF was significantly associated with high tracheal mucus score, high neutrophil proportions in BALF and diagnosis of MA. **Main limitations:** Mycology was only performed in pooled BALF, and environment was not sampled. **Conclusion and clinical importance:** Fungi were significantly more prevalent in the airways of MA horses than SA and/or controls. Fungal detection on TW, either by cytology or culture, was uninformative in a clinical context. Fungal detection by culture (but not cytology) in BALF represents a risk factor for MA.

INTRODUCTION

Asthma in horses is a common cause of respiratory disease and poor performance. This term was proposed to regroup two distinctive phenotypes of inflammatory respiratory diseases: mild-moderate asthma (MA) – previously known as inflammatory airway disease (IAD), and severe asthma (SA) – previously known as recurrent airway obstruction (RAO).[1] Prevalence of SA has been estimated between 14 and 20% within the northern hemisphere,[2,3] and MA was identified in up to 66% of Standardbred racehorses presented for poor performance in France.[4]

Equine asthma of all severities has common clinical presentations (chronic cough, excess mucus, poor performance) but a wide heterogeneity in terms of triggering factors, severity, and pathologic characteristics.[5] However, strong evidence supports the role of exposure to environmental dust in the pathophysiology of both mild-moderate and severe equine asthma.[1] Exposure to inhaled airborne agents, predominantly *via* stabling and provision of hay feeding, was identified as a risk factor for SA.[6] Controlled challenges and exposure

studies have demonstrated an association between the severity of airway neutrophilic response in SA horses and exposure to β-glucan (marker of mold exposure), or with the concentration of mold spores.[7,8] In MA, β-glucan contents of respirable dust have been associated with increased proportions of mast cells in bronchoalveolar lavage fluid (BALF).[9] Moreover, a recent publication showed that horses with fungal elements detected in the tracheal wash (TW) cytology were twice likely to have MA than horses with no fungi.[10] However, a retrospective study on 886 samples showed that cytological evidence of fungal elements in BALF was not a risk factor for respiratory inflammation.[11]

For clinical purposes, questions remain about the most relevant sampling site and methodology for detection of fungal elements in equine airways. Laboratory analytical methods for fungal detection include either cytology or fungal culture of respiratory tract fluids. Poor agreement between those two techniques has previously been reported.[10,12] In TW samples, fungal detection is common, occurring in 55 to 82% of samples depending on methodology,[10,12] rendering questionable the clinical significance of the presence of fungi in TW. Fungal detection in BALF is less common (estimated at 37% in a recent study[12]), with a poor agreement between the two sampling sites.[12] One rationale for performing fungal detection on TW samples is that this sample reflects more accurately the overall exposure of the equine airways to these particles.[10]However, as equine asthma is a disease of the lower respiratory tract,[1] investigating fungal detection in BALF may be a more accurate sampling site regarding the association of mold and development of equine asthma.

With the hypothesis that exposure to fungal elements are a risk factor for equine asthma, the aim of the study was to investigate different combinations of analytical methods/sampling sites in relation to the clinical status. To do so, the objectives were to: 1) determine the prevalence of fungal elements in different respiratory samples (TW or BALF) and methods (cytology or fungal culture), 2) compare the prevalence of fungal elements between groups (control, MA and SA), and 3) determine any eventual association between fungal detection and clinical status.

MATERIALS AND METHODS

Study population

The study included 90 French Standardbred racehorses in active training, sampled between October 2020 and January 2023; and 84 horses referred to the hospital between February 2021 and May 2023 for decreased performance and/or respiratory disease. Minimal sample size was estimated at 92 horses (Epi InfoTM, Cohort and cross-sectional study statistical calculator) in order to be able to detect a significant odd ratio of 3.5. Data concerning housing conditions (indoor/outdoor), bedding (straw, wood shavings), hay forage (dry/soaked/steamed), activity (training/resting), occurrence of poor performance or exercise intolerance, as well as reported respiratory clinical signs (cough, dyspnea, nasal discharge, noise during exercise, increased respiratory effort at rest, epistaxis), were collected. Horses that had received corticotherapy or antimicrobial medication within the two weeks before examination were not included in the study. Horses with signs of systemic illness, other causes of decreased performance or other respiratory pathologies than equine asthma were excluded. The study was approved by the regional Animal Ethic Committee (CERVO-2020-3-V).

Horses were sampled at least 24 hours after the last training or race. Each horse was submitted to a thorough clinical examination by an ECEIM diplomate or resident. If observed, the presence of cough, nasal discharge, nostril flaring at rest, or dyspnea was recorded. Rebreathing pulmonary auscultation was performed and abnormal pulmonary (wheezes/crackles) or tracheal sounds, induction of cough, and delayed recovery following removal of the rebreathing bag were recorded. Venous blood samples were collected for hematology, and additional biochemical analysis when judged clinically necessary.

Respiratory sampling

Horses that were not racing or competing within 10 days following sampling were sedated with detomidine (10 µg/kg IV, Sedomidine, Audevard), with the addition of butorphanol (10-20 µg/kg IV, Torphadine, Dechra) when needed. A nose twitch was used when necessary. For horses racing or competing within 4 to 10 days following investigation, the procedures were achieved with a nose twitch only, without sedation because of

drug testing considerations.

Tracheal wash

A 120 cm long videoendoscope (AOHUA, Medimage off-site; OLYMPUS in-house) was passed through one of the nostrils into the pharynx. The endoscope was then inserted into the proximal trachea, and the presence of blood in the trachea and the mainstem bronchi was graded from 0 to 4 based on a reported scoring system (tracheal blood score, TBS).[13] Tracheal mucus was also scored according to a previously published scale from 0 to 5.[14] A single-use double-lumen sterile catheter (Mila International Inc.) was passed through the canal of the endoscope, and 40 mL of warmed (37°C) sterile saline solution (0.9% NaCl) was infused and retrieved. The aspirated fluid was divided between a sterile dry tube for microbiology (bacteriology and mycology), and a tube containing EDTA for cytological analysis.

Bronchoalveolar lavage

The BAL was performed using a flexible 320 cm long videoendoscope (AOHUA, Medimage off-site; OLYMPUS in-house). After visualization of the carina, the endoscope was inserted into the right main bronchus until wedged. A total of 500 mL (two boluses of 250 mL each) of warmed (37°C) sterile isotonic saline solution was instilled through the endoscope biopsy channel, using 60 mL pre-filled syringes. After each bolus, the liquid was aspirated with the same syringes, and the first 20 mL corresponding to the endoscope channel volume that did not reach the lung was systematically discarded. At the end of lavage of the first lung side, the endoscope instrument channel was cleaned with a 60 mL bolus of sterile isotonic saline; then the endoscope was moved back to the carina and introduced in the contralateral lung and lavage procedure was repeated.

The volume of liquid collected and macroscopic assessment were recorded. A proportional volume of BALF from each lung was pooled in a jar to obtain a pooled BALF.

Endoscopes were sterilized between each horse by flushing the channels and soaking the endoscopes in disinfectant (Hexanios G+R, Anios Laboratories) as per manufacturer's recommendations. Left, right and pooled samples were then placed in a tube containing EDTA for cytology, and another aliquot of pooled samples was placed in a sterile tube for mycology. All samples were kept chilled until handled by the laboratory, and all analyses were performed within 24 hours of sampling.

Cytology

Three hundred microliters of fluid from TW and BALF (left, right and pooled) were cytocentrifuged ($80g$, 10 min) and stained with May-Grünwald-Giemsa (MGG). A differential cell count was performed on 400 cells and the number of each cell type was recorded as a percentage of total nucleated cells, excluding epithelial cells. The presence of fungal particles (spores and hyphae, extracellular or phagocytosed) and bacteria was documented.

Microbiology

Bacteriological examinations were performed as previously described[15] and consisted of direct isolation on selective and non-selective media, Gram staining and plate inoculation after dilution for counting.[16] Mycology testing was performed by direct isolation on selective media as previously described.[12] Samples were incubated for up to 6 days, and identification was performed by biochemical identification (standard API kits, Biomerieux). In order to rule out extrinsic contamination by the equipment, videoendoscopes were tested for fungal contamination. Sampling of each endoscope was performed twice (two months apart) throughout the study. Briefly, the tip of the disinfected videoendoscopes was placed in an empty, sterile, plastic jar. Two 60-mL syringes pre-filled with sterile saline were infused through the biopsy channel into the sterile jar, with the tip of the scope dipping in the solution. The infused solution was then aspirated through the biopsy channel and placed in a sterile tube for mycological culture.

Molecular biology

All qPCR have previously been validated in respiratory samples, based on the NF-U-47600-2 AFNOR norm.[17] Detection of the following viruses was performed in TW: equine herpesvirus (EHV) -1, -2, -4 and -5; equine rhinitis virus A (ERAV) and B (ERBV); equine adenovirus (EAdV) -1 and -2; and equine influenza virus (EIV). Herpesvirus-2, EHV-5 and ERBV were also assessed in the pooled BALF. Ten mL of TW and 25 mL of BALF were centrifuged (800 g; 20 min; 4 degC), and pellets were re-suspended with 1 mL of supernatant. Extraction of viral nucleic acids and virus specific qPCR were performed using previously reported assays.[18]

Case definition

Based on the previous consensus statement,[1] a diagnosis of mild-moderate asthma (MA) was made based on the presence of clinical signs (poor performance, chronic occasional coughing, or both), evidence of airway inflammation in BALF cytology, presence of significant amount of tracheal mucus detected by endoscopy (grade > 2/5), and the exclusion of severe asthma (SA) based on history and clinical signs. For BALF cytology, the 'less restrictive' cut-off was used: neutrophils > 10%, mast cells > 5% and eosinophils > 5% in individual (left and right) BALF,[1,19] and neutrophils > 9%, mast cells > 3% and eosinophils > 3% in pooled BALF.[20] A diagnosis of SA was made based on history, presence of increased respiratory effort at rest, and moderate to severe neutrophilia in BALF (> 25%) in horses of middle to advanced age (> 7 years old).[1] Horses without coughing, nasal discharge, or increased respiratory effort, with normal BALF cytology from both lungs, and with a negative bacterial culture from TW were classified as controls (CTL). Cytology was classified as positive when at least one fungal element (spore, hyphae) was observed on the slide (TW, individual or pooled BALF). Fungal culture was classified as positive when one or more colonies were observed (and identified) in a sample (TW or pooled BALF).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software. A Chi-square test (or a Fisher's exact test when necessary) was performed to compare the final prevalences among groups according to each sample modality. Association between fungal detection and qualitative variables (extrinsic factors, clinical examination, clinical status) was evaluated with a Chi-square test, as well as odds ratio, sensitivity, specificity and predictive values**.** Association between fungal detection and quantitative variables (neutrophil proportions) was determined using Cochran-Mantel-Haenszel test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Study population

Among the 174 horses sampled, 3 horses were excluded because samples were uninterpretable on reception at the laboratory, and 16 were excluded from the study because of respiratory issues unrelated to equine asthma: 10 with infectious pneumonia, 5 with exercise-induced pulmonary haemorrhage (EIPH) and 1 with interstitial pneumonia. Overall, 155 horses were included. The population was composed of 85 Standardbred racehorses (54.8%), 20 sport horses (12.9%), and 50 leisure horses (32.3%). Horses were aged from 2 to 25 years (median 6 years), with a total of 38 entire males (24%), 60 geldings (39%) and 57 females (37%).

Of these, 85 horses were diagnosed with MA, 35 horses were SA and 35 horses were considered controls (CTL). Amongst the MA group, 29.4% (25/85) exhibited mast cell inflammation only, 2.4% (2/85) eosinophilic inflammation only, 43.5% (37/85) neutrophilic inflammation only, 11.8% (10/85) increased tracheal mucus score only and 12.9% (11/85) had a mixed pattern amongst the four inclusion criteria. All horses had a TBS of 0.

Bacterial and viral detection on airway samples

Bacteria were observed in the TW of 11 horses. *Streptococcus equi*subsp *zooepidemicus* (20 000 – 12 000 000 CFU/mL) was identified in 6 horses, 4 horses with MA and 2 horses with SA. *Actinobacillus haemolyticus* (10 000 – 2 000 000 CFU/mL) was identified in 5 samples, 3 horses with MA and 2 horses with SA. *Mycoplasma*

spp was detected in the TW of 34.3% (57/155) of all horses, with 31.6% (12/35) of CTL horses, 38.8% (33/85) of MA horses and 28.6% (12/35) of SA horses respectively.

Herpesvirus-4 was detected in TW of 2 horses (1.3%), both were in MA group. Herpersvirus-2 and EHV-5 were detected in TW of 30.3% (47/155) and 38.1% (59/155) of all horses respectively. Herpesvirus-2 was identified in 37.2% (13/35) of CTL horses, 28.2% (24/85) of MA horses and 34.3% (10/35) of SA horses. On the other hand, EHV-5 was identified in 40% (14/35) of CTL horses, 42.4% (36/85) of MA horses and 25.7% (9/35) of SA horses.

Equine rhinitis virus B was detected in TW of 38.6% (59/153) of horses, with 42.4% (14/33) of CTL horses 38.8% (33/85) of MA horses and 34.3% (12/35) of SA horses. Herpersvirus-1, ERAV, EAdV-1 and -2 and EIV were not detected in any TW sample.

In BALF, EHV-2 was identified in 8.4% (13/154) of horses, with 13.1% (11/84) in the MA group and 5.7% (2/35) in the SA group. Equine herpesvirus-5 was identified in 7.1% (11/154) of all samples, with 2.9% (1/35) in the CTL group, 9.5% (8/84) in the MA group and 5.7% (2/35) in the SA group. Equine rhinitis virus B was not detected in any BALF sample.

Fungal detection in TW

Overall, a positive TW cytology was obtained in 75.5% (117/155) of horses (Table 1). Proportion of samples positive for fungi on cytological examination was significantly lower in the SA group than in CTL ($P = 0.007$) and MA ($P < 0.001$) groups (Table 1; Figure 1A). Similarly, the proportion of fungi phagocytosed by macrophages was significantly lower in the SA group than the CTL ($P = 0.02$) and MA ($P = 0.01$) groups. The proportion of spores or hyphae (phagocytosed or not) was not significantly different between groups.

Overall, a positive TW mycology was obtained in 81.9% (127/155) of all horses. Proportion of samples positive for fungi by culture was significantly higher in the MA group than the CTL group ($P = 0.005$) (Figure 1B). In the TW, 17 fungal *genera* were identified, with the most commonly isolated *genera* being *Aspergillus* sp. (80/127, 63%), *Penicillium* sp. (66/127, 52%), and *Chrysosporium* sp. (45/127, 35.4%), alone or in combination with other fungi (Table S1). Culture allowed identification of 7 species of *Aspergillus* sp., alone or in combination with other species: *A. nidulans* (n=35), *A. fumigatus* (n=29), *A. niger* (n=15), *A. glaucus* (n=14), *A. terreus* (n=6), *A. versicolor* (n=6) and *A. flavus* (n=2).

The number of fungal *genera* concomitantly identified according to samples and group is available in Figure 2. *Aspergillus* sp. was identified in 54.2% (13/24) of CTL horses, 68.4% (52/76) of MA horses and in 55.6% (15/27) of SA horses, with no significant difference between groups. Proportions positive for *Penicillium* sp. were not significantly different between groups; this genus was found in 66.7% (16/24) of CTL horses, in 47.4% (36/76) of MA horses and in 51.9% (14/27) of SA horses. Similarly, proportions of *Chrysosporium* sp. did not differ significantly between groups, with 33.3% (8/24) in CTL horses, 36.8% (28/76) in MA horses and 33.3% (9/27) in SA horses.

Fungal detection in BALF

Overall, a positive BALF cytology was obtained in 18.1% (28/155) of all horses (Table 2).

Proportion of positive samples for fungi on cytological examination was significantly lower in the SA group than the MA group ($P = 0.02$) but not the CTL group (Table 2; Figure 1C).

No significant difference was found between CTL and MA groups. Proportions of fungi phagocytosed by macrophages and proportions of spores or hyphae (phagocytosed or not) were no significantly different between groups.

Overall, a positive BALF mycology was obtained in 23.9% (37/155) of horses. Proportion of positive samples for fungi by culture was significantly higher in the MA group than the CTL group ($P = 0.008$) (Figure 1D).

In BALF, 8 fungal *genera* were identified (Table S2), with the most commonly isolated *genera* being *Aspergillus* sp. (56.8%, 21/37), *Aureobasidium* sp. (27%, 10/37) and *Penicillium* sp. (21.6%, 8/37). *Aure-*

obasidium sp. was however not detected in any TW sample. Culture allowed identification of 6 species of *Aspergillus* sp., alone or in combination with other species: *A. fumigatus* (n=8), *A. nidulans* (n=7), *A. glaucus* (n=7), *A. niger* (n=3), *A. versicolor (n=2) and A. terreus* (n=1).

Aspergillus sp. was identified in 100% (3/3) of CTL horses, 55.6% (15/27) of MA horses and in 42.9% (3/7) of SA horses, with no significant difference between groups. Proportions of*Aureobasidium* sp. was not significantly different between groups; this *genus* was found in 33.3% (1/3) of CTL horses, in 33.3% (9/27) of MA horses and in 0% (0/7) of SA horses. Similarly, proportions of *Penicillium* sp. did not differ significantly between groups, with 0% (0/3) in CTL horses, 14.8% (4/27) in MA horses and 57.1% (4/7) in SA horses.

Association between fungal detection and extrinsic factors

The likelihood of finding fungal elements in TW and BALF by culture and cytology was not significantly associated with either housing (pasture, straw or shavings) or feeding (dry, wet or sterilized hay). No significant associations were found between fungal detection in TW or BALF by culture or cytology and identification of bacteria in TW, identification of *Mycoplasma* spp in TW or identification of virus in TW.

Horses where EHV-2 was detected in BALF had 2-fold higher odds of exhibiting a positive fungal culture in BALF (Table 3).

Association between fungal detection and clinical examination

The prevalence of fungal elements either in TW or BALF by culture was not significantly associated with clinical signs (cough, nasal discharge, dyspnea, wheezes or crackles). Horses with cough were significantly less likely to exhibit fungi by cytology in TW and BALF (Table 3). Horses with a tracheal mucus score >2 were significantly less likely to exhibit fungi by cytology in TW. Horses with a tracheal mucus score >2 had 2-fold higher odds of having a positive fungal culture in BALF. Horses with an increased proportion of neutrophils in BALF had 2-fold higher odds of exhibiting fungi by culture in BALF. The magnitude of inflammatory neutrophilic reaction, when present (> 10%), was however not significantly associated with fungal detection in BALF (Table S3). No association was found between fungal detection by cytology in BALF and the proportion of neutrophils, nor between fungal detection (by culture or cytology) in BALF and the proportion of mast cells.

Association between fungal detection and clinical status

No significant association was observed between clinical status (MA*vs* . CTL) of horses and positive cytology in TW. Horses with SA were significantly less likely to exhibit a positive cytology in TW compared with CTL, with a substantial sensitivity and specificity, as well as a moderate positive predictive value (PPV) and a substantial negative predictive value (NPV) (Table 4, Figure 1A). Horses with MA had almost 8-fold higher odds of positive cytology in TW compared with SA horses, with a substantial sensitivity, a good specificity, and a moderate PPV and a good NPV (Table 4, Figure 1A).

No significant association was observed between status (SA *vs* . either CTL or MA) of horses and positive mycology culture in TW. Horses with MA had almost 4-fold higher odds of positive mycology in TW compared with CTL horses, with a moderate sensitivity, a substantial specificity, and a fair PPV and a good NPV (Table 4, Figure 1B).

No significant association was observed between status (MA *vs.*CTL or SA *vs.* CTL) of horses and positive cytology in BALF. Horses with MA had 5-fold higher odds of positive cytology in BALF compared with SA horses, with a fair sensitivity, a good specificity, and a good PPV and a poor NPV (Table 4, Figure 1C).

Horses with MA had almost 5-fold higher odds of positive mycology in BALF compared with CTL horses, with a poor sensitivity, a good specificity, and a good PPV and a poor NPV (Table 4, Figure 1D).

DISCUSSION

Our study demonstrated that fungal elements were commonly present in the trachea, with a positive TW mycology obtained in 81.9% of samples and fungal elements present upon cytological examination of TW in 75.5% of samples. In contrast, fungal detection was scarce in the BALF, with fungal elements found upon cytological examination in 18.1% of samples and a positive BALF mycology culture obtained in 23.9% of samples. The results from this study partially supported our hypothesis that exposure to fungal elements was a risk factor for asthma, since only horses with MA had almost 5-fold higher odds of positive mycology in BALF compared with CTL horses. Furthermore, horses with an increased score of tracheal mucus were more likely to exhibit positive fungal culture in BALF, as well as horses with an increased proportion of neutrophils in BALF.

Analytical methods of fungal detection

In order to refine clinical interpretation, potential cytological markers have been investigated in our study, such as identification of type of fungal elements (spores or hyphae) and whether they were phagocytosed or not. As spores represent a dormancy form and hyphae a growing form of fungi, the latter have previously been evaluated in the context of MA.[10] Our results showed that identification of growth as well as phagocytosis of fungal elements is not significantly correlated with either clinical signs, inflammation or diagnosis of equine asthma; thus precluding the use of these as biological markers of occurrence or severity of asthma in horses.

A high prevalence of fungal detection by cytology in TW of non-asthmatic horses has been reported previously,[10,21,22] and also confirmed in the current study. Moreover, the prevalence of fungal detection by cytology in BALF of CTL and MA horses drives to the conclusion that this method is not relevant for the investigation of fungi elements in athletic horses. Indeed, horses with a positive cytology in TW or BALF were less likely to exhibit cough, because of the very large proportions of fungi detected by this method among control horses with no clinical signs. However, the results of the current study did identify a significant association between fungal detection by cytology in BALF and SA. Severe asthmatic horses were indeed prone to exhibit fewer fungal elements compared to moderate asthmatics. This finding concurs with a previous study in which intracellular fungal-like particles were less commonly observed in the macrophages of severe asthmatic horses exposed to hay, compared to healthy horses.[23] The authors suggested that fungal particles were trapped more proximally in the respiratory tract, due to increased mucus and bronchoconstriction. An alternative hypothesis would be a decreased phagocytic capacity of macrophages of horses with SA, as previously demonstrated *ex vivo* .[24]

Prevalence of fungal detection

A large number of fungal *genera* were identified in this study, with *Aspergillus* sp. and *Penicillium* being the most commonly found in respiratory fluids. While similar *genera* have been reported in previous studies,[10,25] up to 5 different *genera* or up to 5 different species of*Aspergillus* sp. could be concomitantly identified in the airways of a single horse. It has previously been documented that*Penicillium* , *Aspergillus* sp., and *Muroraceaegenera* were airborne, ubiquitous and frequently found in the air of stables[26] while *Aspergillus* sp. is the most commonly isolated fungi in hay.[12,27] Detection of these fungal elements in the airways may simply reflect the environment in which the horse was located before and during sample collection.[10,21,28] Indeed, the authors of a recent publication identified the fungal microbiota present along the upper and lower respiratory tract of healthy horses, and showed that fungal microbiota of the pharynx was similar to that of the trachea in healthy horses, indicating that the pharynx is potentially a major source of microbes that translocate to the lower respiratory tract.[25] Even though an association between fungal content in hay samples and fungal elements in the airways of horses has not been identified,[12] other sources of contamination in horses' stables remain to be investigated.

In the current study, the *genus Aureobasidium* sp. was found in BALF only (and not in TW) of 10 horses (9 MA, 1 CTL). In horses, few anecdotal studies reported the detection of *Aureobasidium* sp. in fungal microbiota of the conjunctiva of clinically healthy horses,[29] in paranasal sinus of healthy and diseased horses[30] and through ITS2 gene sequencing (mycobiota) in BALF of horses.[31] However, the existence of an immune-mediated hypersensitivity reaction leading to interstitial pneumonitis due to *Aureobasidium pullulans* has

been reported in the human literature.[32] Further studies are required to confirm the possible pathogenicity of *Aureobasidium* sp. and determine whether specific *genera* would present more risk than others for the development of equine asthma.

Association between fungal detection and other infectious agents

To our knowledge, this is the first study to record detection of multiple types of infectious agents (bacterial, viral, fungal and*Mycoplasma* spp) in both TW and BALF, and to examine their associations with equine asthma. Isolation of bacteria in TW has previously been associated with lower airway inflammation.[33–36] However, due to a very low prevalence of positive bacterial samples in the present study, it was not possible to confirm this association with moderate or severe equine asthma. Alternatively, frequent co-detection of fungal elements and other infectious agents such as viruses or mycoplasms has been highlighted in this study. *Mycoplasma* spp were recently found to be detected in 19.4% of TW samples from horses with respiratory diseases,[37] although their detection in TW was not significantly different between groups (CTL, MA, SA) in the current study. The potential implication of mycoplasms in equine asthma remains to be determined, especially by quantitative investigation as per other bacteria in TW.

Our results indicated a significant positive association between fungal detection by culture in BALF and detection of EHV-2 in BALF. So far, the evidence of a potential role of respiratory viruses is controversial; some studies report an association between MA and genome detection by PCR for EHV-2, EHV-5 and ERBV,[18,38] while others do not.[39,40] There has been speculation that these viruses may act as immunosuppressive agents, predisposing to other infections,[41] or may be involved in persistent, chronic fatigue syndromes.[42] Larger multicentric studies would be required to determine the possible synergy between viruses, bacteria, fungi, particles etc. (interactome) in the pathophysiology of equine asthma.

Association between fungal detection and inflammatory response

A significant positive association was found between fungal detection by culture in BALF and a high tracheal mucus score, as well as with a high percentage of neutrophils in BALF. When present (>10%) the magnitude of neutrophilic response was however not significantly associated with the prevalence of mold in BALF. Tracheal mucus accumulation and neutrophil proportions are evidence of airway inflammation, but without specific cause.[5]

Fungal exposure and its association with SA have frequently been documented,[8,43,44] but studies examining fungal association with MA are still scarce. One study found that horses with fungal elements observed in TW cytology had twice the odds of having IAD than horses without fungi.[10] There is also little information to date concerning the association between antigenic triggers and specific MA phenotypes.[45] Neutrophilic airway inflammation in BALF of racing Thoroughbreds has been correlated with both respirable dust and endotoxin exposure, while mast cell inflammation in BALF has been associated with β-glucan exposure.[9] Interestingly, our results tend to demonstrate the lack of association between fungal detection in the airways and non-neutrophilic forms (mast-cell and eosinophilic) of pulmonary inflammation. These elements suggest that fungal detection in the airways is not linked to a specific immune response.

LIMITATIONS

Fungal detection in BALF was performed on pooled BALF only, and not on individual right and left BALF, which could have led to an overall lower prevalence of fungal detection by culture in BALF. In a previous study, while 100% of horses with "positive" pooled BALF were also positive in left and/or right BALF, 14.6% of horses with "negative" pooled BALF were positive in left and/or right BALF.[12]

Beyond detection of fungi in TW or BALF, quantification of the fungal load in the airways also needs to be further investigated. Cytological investigations of fungal elements were limited to qualitative dichotomy (presence or absence) only. Quantification of fungal-like intracellular particles by cytology or total fungal load quantification by qPCR assay (18S rRNA gene) have recently been proposed, but these methods are yet to be validated.[23] Absolute quantification by successive dilutions (as performed for bacteria in TW for instance)[16] were laborious, unrepeatable (data not shown) and ultimately unsuccessful for fungal cultures.

However, establishment of a "pathological" cut-off, possibly through molecular biology, could however help in the future for differentiating true fungal infection from environmental contamination.

This study focused on fungi detection in airways samples, while ambient airborne samples or component of horses' environment (bedding, food) were not collected. No association between fungal content in hay samples and fungal elements in the airways of horses was detected in a previous study.[12] It would however have been interesting to collect respirable and inhaled particulate samples in the horse's breathing zone, as previously described.[47,48] The relevance of such sampling, factually excluding horses referred to the hospital for consultation, is however limited to field studies only, most frequently focusing on MA rather than SA.

CONCLUSION

Overall, the prevalence of fungal detection was found to be significantly higher in the airways of MA horses than SA and/or CTL horses. Due to the high proportion of positive fungal detection, by either cytology or culture, in TW of CTL horses, investigations based on this type of samples were found to be uninformative in a clinical context. On the other hand, fungal detection by culture (but not by cytology) in BALF has been identified as a significant risk factor for mild-moderate asthma in horses.

TABLES

	Fungal elements	Fungal elements	Spores	Spores	Hyphae	Hyphae
	Overall prevalence	Phagocytosed	Proportion among total	Phagocytosed	Proportion among total	Phagocytosed
General population	75.5 % (117/155)	66.7% (78/117)	87.2% (102/117)	71.6% (73/102)	74.4% (87/117)	60.9% (53/87)
CTL	77.1% [a] (27/35)	74.1% [a] (20/27)	92.6% (25/27)	76.0% (19/25)	81.5% (22/27)	63.7% (14/22)
MA	87.1% [a] (74/85)	70.3% [a] (52/74)	87.8% (65/74)	73.8% (48/65)	73.0%% (54/74)	64.8% (35/54)
SA	45.7% [b] (16/35)	37.5% [b] (6/16)	75% (12/16)	50.0% (6/12)	68.8% (11/16)	36.4% (4/11)

Table 1: Prevalence of positive fungal cytology and phagocytosis of fungal elements in tracheal wash according to clinical status

CTL: control group; MA: mild-moderate asthma group; SA: severe asthma group

Different letters correspond to significant differences between groups ($P < 0.05$).

	Fungal elements	Fungal elements	Spores	Spores	Hyphae
	Overall prevalence	Phagocytosed	Proportion among total	Phagocytosed	Proportion among
General population	18.1 % (28/155)	89.3% (25/28)	100.0% (28/28)	89.3% (25/28)	50.0% (14/28)
CTL	17.1% [a,b] (6/35)	83.3% (5/6)	100.0% (6/6)	83.3% (5/6)	33.3% (2/6)
MA	23.6% [b,c] (20/85)	90.0% (18/20)	100.0% (20/20)	90% (18/20)	36.4% (10/20)
SA	5.7% [a] (2/35)	100.0% (2/2)	100.0% (2/2)	100.0% (2/2)	100.0% (2/2)

Table 2: Prevalence of positive fungal cytology and phagocytosis of fungal elements in bronchoalveolar lavage fluid according to clinical status

CTL: control group; MA: moderate asthma group; SA: severe asthma group

Different letters or different symbols correspond to significant differences between groups ($P < 0.05$).

Variable	Method of detection	Site of detection	Odds ratio	Lowe
EHV-2 detection in BALF	Culture	BALF	2.42	1.03
Cough	Cytology	TW	0.33	0.16
Cough	Cytology	BALF	0.27	0.11
Tracheal mucus score >2	Cytology	TW	0.19	0.09
Tracheal mucus score >2	Culture	BALF	2.26	1.03
Increased proportion of neutrophils (>10%) in BALF	Culture	BALF	2.23	1.06

Table 3: Association between fungal detection and extrinsic factors/clinical examination

BALF: bronchoalveolar lavage fluid, TW: tracheal wash, CI: confidence interval, EHV-2: Equine Herpesvirus-2

Variables were displayed in the table only when $p < 0.05$.

Variable	Clinical status	Odds ratio	Lower 95% CI	Upper 95% CI	Sensitivity	Speci
Positive cytology in TW	CTL	ref	-	-	-	-
	SA	0.25	0.09	0.70	0.70	0.63
Positive cytology in TW	SA	ref	-	-	-	-
	MA	7.99	3.14	19.74	0.63	0.82
Positive mycology in TW	CTL	ref	-	-	-	-
	MA	3.87	1.37	10.08	0.55	0.76
Positive cytology in BALF	SA	ref	-	-	-	-
	MA	5.08	1.30	22.86	0.34	0.91
Positive mycology in BALF	CTL	ref	-	-	-	-
	MA	4.97	1.49	16.38	0.36	0.90

Table 4: Association between fungal detection and clinical status

CTL: control group, MA: mild-moderate asthma group, SA: severe asthma group, CI: confidence interval, PPV: positive predictive value, NPV: negative predictive value

Variables were displayed in the table only when $p < 0.05$.

LIST OF FIGURES

Figure 1: Proportions of positive fungal detection depending on clinical status and sampling site. A: TW by cytology, B: TW by culture, C: BALF by cytology, D: BALF by culture

TW: Tracheal wash; BALF: bronchoalveolar lavage fluid; CTL: control group; MA: mild-moderate asthma group; SA: severe asthma group

Figure 2: Number of fungi genera identified in respiratory samples according to clinical status. A: TW, B: BALF

TW: Tracheal wash; BALF: bronchoalveolar lavage fluid; CTL: control group; MA: mild-moderate asthma group; SA: severe asthma group

LIST OF SUPPLEMENTARY ITEMS

Table S1: Relative proportions of fungal genera identified in tracheal wash

Table S2: Relative proportions of fungal genera identified in bronchoalveolar lavage fluid

Table S3: Association between fungal detection by culture in BALF and neutrophil cells percentage in the TW.

BALF: bronchoalveolar lavage fluid

REFERENCES

1. Couëtil LL, Cardwell JM, Gerber V, Lavoie JP, Léguillette R, Richard EA. Inflammatory Airway Disease of Horses—Revised Consensus Statement.*J Vet Intern Med* . 2016;30(2):503–515.

2. Hotchkiss JW, Reid SW, Christley RM. A survey of horse owners in Great Britain regarding horses in their care. Part 2: Risk factors for recurrent airway obstruction. *Equine Vet J* . 2007;39(4):301–308.

3. Ireland JL, Christley RM, McGowan CM, Clegg PD. Prevalence of and risk factors for recurrent airway obstruction in geriatric horses and ponies. *Equine Vet J* . 2015;47:25.

4. Richard EA, Fortier GD, Pitel PH, Dupuis MC, Valette JP, Art T, et al. Sub-clinical diseases affecting performance in Standardbred trotters: Diagnostic methods and predictive parameters. *Vet J* . 2010;184(3):282–289.

5. Couëtil L, Cardwell JM, Léguillette R, Mazan M, Richard E, Bienzle D, et al. Equine asthma: current understanding and future directions.*Front Vet Sci* . 2020;7:450.

6. Pirie RS. Recurrent airway obstruction: a review. *Equine Vet J* . 2014;46(3):276–288.

7. Pirie RS, Dixon PM, McGorum BC. Endotoxin contamination contributes to the pulmonary inflammatory and functional response to*Aspergillus fumigatus* extract inhalation in heaves horses.*Clin Exp Allergy* . 2003;33(9):1183–1189.

8. Pirie RS, Collie DDS, Dixon PM, McGorum BC. Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 2: Effects of inhaled HDS on control and heaves horses.*Equine Vet J* . 2010;34(4):337–342.

9. Ivester KM, Couëtil LL, Moore GE. An observational study of environmental exposures, airway cytology, and performance in racing thoroughbreds. *J Vet Intern Med* . 2018;32(5):1754–1762.

10. Dauvillier J, Woort F, Van Erck-Westergren E. Fungi in respiratory samples of horses with inflammatory airway disease. *J Vet Intern Med* . 2019;33(2):968–975.

11. Venture P, Toquet MP, Courouce A, Richard EA. Cytological profiles of equine bronchoalveolar lavage fluid: a retrospective study (2013-2015). Proceedings of the World Equine Airway Symposium, Copenhagen, Denmark, 2017.

12. Lemonnier LC, Courouce A, Cessans M, Petit L, Cardwell JM, Barbazanges P, et al. Detection of fungi in the airways of horses according to the sample site: a methodological study. *Vet Res Commun* . 2024;48(1):345-355.

13. Hinchcliff KW, Jackson MA, Brown JA, Dredge AF, O'Callaghan PA, McCaffrey JP, et al. Tracheobronchoscopic assessment of exercise-induced pulmonary hemorrhage in horses. *Am J Vet Res* . 2005;66(4):596–598.

14. Gerber V, Straub R, Marti E, Hauptman J, Herholz C, King M, et al. Endoscopic scoring of mucus quantity and quality: observer and horse variance and relationship to inflammation, mucus viscoelasticity and volume. *Equine Vet J* . 2010;36(7):576–82.

15. Richard EA, Fortier GD, Lekeux PM, Erck EV. Laboratory findings in respiratory fluids of the poorly-performing horse. *Vet J* . 2010;185(2):115–122.

16. Maillard K, Richard EA, Kuhnert P, Fortier G, Leon A, Pitel PH. Isolation of *Nicoletella semolina* from equine tracheal washes.*J Equine Vet Sci.* 2013;33(7):561–564.

17. Hue ES, Fortier CI, Laurent AM, Quesnelle YF, Fortier GD, Legrand LJ, et al. Development and validation of a quantitative PCR method for equid herpesvirus-2 diagnostics in respiratory fluids. *J Vis Exp* . 2016;(109):53672.

18. Doubli-Bounoua N, Richard EA, Leon A, Pitel PH, Pronost S, Fortier G. Multiple molecular detection of respiratory viruses and associated signs of airway inflammation in racehorses. *Virol J* . 2016;13(1):197.

19. Depecker M, Richard EA, Pitel PH, Fortier G, Leleu C, Courouce-Malblanc A. Bronchoalveolar lavage fluid in Standardbred racehorses: Influence of unilateral/bilateral profiles and cut-off values on lower airway disease diagnosis. *Vet J.*2014;199(1):150–156.

20. Hermange T, Le Corre S, Bizon C, Richard EA, Courouce A. Bronchoalveolar lavage fluid from both lungs in horses: Diagnostic reliability of cytology from pooled samples. *Vet J* . 2019;244:28–33.

21. Beech J. Cytology of tracheobronchial aspirates in horses. *Vet Pathol* . 1975;12(3):157–164.

22. Sweeney CR, Humber KA, Roby KA. Cytologic findings of tracheobronchial aspirates from 66 thoroughbred racehorses. *Am J Vet Res* . 1992;53:1172–1175.

23. Di Pietro R, Dubuc V, Manguin E, Giroux-Lafond R, Bedard C, Boivin R, et al. Characterization of fungal exposure and dectin-1 expression in healthy horses and horses with severe asthma. *Am J Vet Res* . 2022;83(6)

24. Hue E., Orard M., Toquet M.-P., Courouce A., Bizon-Mercier C., Moore-Colyer M., et al. In vitro stimulation of alveolar macrophages from severe asthmatic horses. 37th Annual VCRS Symposium, Caen, France, 2019.

25. Bond S, McMullen C, Timsit E, Leguillette R. Topography of the respiratory, oral, and guttural pouch bacterial and fungal microbiotas in horses. *J Vet Intern Med* . 2023;37(1):349–360.

26. Nardoni S, Mancianti F, Sgorbini M, Taccini F, Corazza M. Identification and seasonal distribution of airborne fungi in three horse stables in Italy. *Mycopathologia* . 2005;160(1):29–34.

27. Kotimaa MH, Husman KH, Terho EO, Mustonen MH. Airborne molds and actinomycetes in the work environment of farmer's lung patients in Finland. *Scand J Work Environ Health.* 1984;10(2):115–119.

28. Higgins JC, Pusterla N. Fungal Pneumonia in Horses. *Clinical Techniques in Equine Practice* . 2006;5(3):218–224.

29. de Sousa ME, Araujo MA, Mota RA, Porto WJ, Souza AK, Dos Santos JL, et al. Fungal microbiota from ocular conjuctiva of clinically healthy horses belonging to the Military Police Cavalry of Alagoas. *Braz J Microbiol.* 2011;42(3):1151–1155.

30. Beste KJ, Lawhon SD, Chamoun-Emanuelli AM, Duff AH, Coleman MC, Griffin CE, et al. Culture-independent and dependent evaluation of the equine paranasal sinus microbiota in health and disease. *Equine Vet J* . 2020;52(3):455–463.

31. Bond SL, Workentine M, Hundt J, UCVM Class of 2019, Gilkerson JR, Leguillette R. Effects of nebulized dexamethasone on the respiratory microbiota and mycobiota and relative equine herpesvirus-1, 2, 4, 5 in an equine model of asthma. *J Vet Intern Med* . 2020;34(1):307–321.

32. Temprano J, Becker BA, Hutcheson PS, Knutsen AP, Dixit A, Slavin RG. Hypersensitivity pneumonitis secondary to residential exposure to*Aureobasidium pullulans* in 2 siblings. *Ann Allergy Asthma Immunol* . 2007;99(6):562–6.

33. Christley RM, Hodgson DR, Rose RJ, Hodgson JL, Wood JL, Reid SW. Coughing in Thoroughbred racehorses: risk factors and tracheal endoscopic and cytological findings. *Vet Rec* . 2001;148(4):99-104

34. Wood JL, Newton JR, Chanter N, Mumford JA. Inflammatory airway disease, nasal discharge and respiratory infections in young British racehorses. *Equine Vet J* . 2005;37(3):236–242.

35. Cardwell JM, Smith KC, Wood JL, Newton JR. Infectious risk factors and clinical indicators for tracheal mucus in British National Hunt racehorses. *Equine Vet J.*2014;46(2):150–155.

36. Manguin E, Pepin E, Boivin R, Leclere M. Tracheal microbial populations in horses with moderate asthma. *J Vet Intern Med* . 2020;34(2):986–995.

37. Martineau M, Castagnet S, Kokabi E, Tricot A, Jay M, Leon A, et al. Detection of *Mycoplasma* spp. in horses with respiratory disorders. *Equine Vet J* . 2023;55(5):747–754.

38. Fortier G, Van Erck E, Fortier C, Richard E, Pottier D, Pronost S, et al. Herpesviruses in respiratory liquids of horses: Putative implication in airway inflammation and association with cytological features. *Vet Microbiol* . 2009;139(1–2):34–41.

39. Back H, Penell J, Pringle J, Isaksson M, Roneus N, Treiberg Berndtsson L, et al. A longitudinal study of poor performance and subclinical respiratory viral activity in Standardbred trotters. *Vet Rec Open* . 2015;2(1):e000107.

40. Couetil L, Ivester K, Barnum S, Pusterla N. Equine respiratory viruses, airway inflammation and performance in Thoroughbred racehorses. *Vet Microbiol* . 2021;257:109070.

41. Nordengrahn A, Rusvai M, Merza M, Ekstrom J, Morein B, Belak S. Equine herpesvirus type 2 (EHV-2) as a predisposing factor for*Rhodococcus equi* pneumonia in foals: prevention of the bifactorial disease with EHV-2 immunostimulating complexes. *Vet Microbiol* . 1996;51(1–2):55–68.

42. Leon A, Fortier G, Fortier C, Freymuth F, Tapprest J, Leclercq R, et al. Detection of equine herpesviruses in aborted foetuses by consensus PCR. *Vet Microbiol* . 2008;126(1–3):20–29.

43. McGorum BC, Dixon PM, Halliwell RE. Responses of horses affected with chronic obstructive pulmonary disease to inhalation challenges with mould antigens. *Equine Vet J* . 1993;25(4):261–7.

44. Pirie RS, Collie DDS, Dixon PM, McGorum BC. Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma): Dust particulates in equine heaves. *Clin Exp Allergy* . 2003;33(5):676–683.

45. Bond S, Leguillette R, Richard EA, Couetil L, Lavoie JP, Martin JG, et al. Equine asthma: Integrative biologic relevance of a recently proposed nomenclature. *J Vet Intern Med* . 2018;32(6):2088–1098.

46. Stewart AJ, Cuming RS. Update on fungal respiratory disease in horses. *Vet Clin North Am Equine Pract* . 2015;31(1):43–62.

47. Olave CJ, Ivester KM, Couetil LL, Kritchevsky JE, Tinkler SH, Mukhopadhyay A. Dust exposure and pulmonary inflammation in Standardbred racehorses fed dry hay or haylage: A pilot study. *Vet J* . 2021;271:105654.

48. Ivester KM, Smith K, Moore GE, Zimmerman NJ, Couetil LL. Variability in particulate concentrations in a horse training barn over time.*Equine Vet J* . 2012;44(S43):51–56.